A NOVEL NITROREDUCTASE AND THERAPEUTIC USES THEREFOR

RELATED APPLICATIONS

This application claims priority from United States Application Nos. 60/080,917, filed April 6, 1998, and 60/081,778, filed April 14, 1998, the entire contents of both of which are hereby incorporated by reference herein in their entirety.

FIELD OF INVENTION

The present invention relates to nitroreductases, nucleic acids encoding nitroreductases, microaerophilic bacteria from which such nitroreductases may be isolated, conjugates of targeting compounds and nitroreductases and methods of using same.

BACKGROUND

Metronidazole (Mtz) [1-(2-hydroxyethyl)-2-methyl-5-nitroimidazole] is a key component of combination therapies that are widely used against *Helicobacter pylori* (Malfertheiner *et al.*, 1997), a microaerophilic, Gram-negative pathogen that is highly specific for the human gastric mucosa. *H. pylori* tends to establish chronic and often life-long infections that constitute a major cause of peptic ulcer disease and an important risk factor for gastric cancer, one of the most common malignancies worldwide (Correa, 1996). Most residents of developing countries are infected with *H. pylori* (Taylor and Parsonnel, 1995); this situation is ascribed to poor sanitation, which results in frequent exposure to the pathogen. In the U.S. and Western Europe, the prevalence of infection is generally lower, and is correlated with socioeconomic status and age: approximately half of older adults but less than one-tenth of young children in these industrialized societies are *H. pylori*-infected (Taylor and Parsonnel, 1995; Dunn *et al.*, 1997).

Mtz resistance (Mtz^R) is an important variable in the treatment of *H. pylori* infections, indeed its presence markedly reduces the efficiency of Mtz-containing treatment regimens (Chiba *et al.*, 1992; Graham *et al;* 1992). The incidence of Mtz^R also varies geographically with half or more of *H. pylori* strains from developing countries and approximately 10-30% of strains from the US and Western Europe being Mtz^R (Dunn *et al.*, 1997) Veldhuyzen van Zanten *et al.*, 1997). The incidence of Mtz^R among *H. pylori* isolates generally parallels the level of Mtz usage in a particular society. Thus, it is parsimonious to imagine that many of the *H. pylori* strains currently resistant to Mtz reflect the frequent use of Mtz and related nitroimidazoles for treatment of anaerobic and protozoan infections, but in dosing regimens that generally do not eliminate Mtz^S *H. pylori* from an infected person. (Grunberg and Titsworth, 1974; Hoff and Sticht-Groh, 1984; Edwards, 1993). Any inhibition of *H. pylori* growth during such periods of Mtz therapy would enrich or select for Mtz^R strains.

The basis for susceptibility of wild-type *H. pylori* to Mtz and the mechanisms of resistance have been of interest and concern since the early days of *H. pylori* research (see, for example, McNulty *et al.*, 1985; Glupczynski and Burette, 1990). Well-studied model organisms such as *Pseudomonas aeruginosa* and *Escherichia coli,* which are aerobic or facultatively anaerobic, are Mtz^R, whereas many anaerobics and microaerophiles are susceptible to Mtz (Mtz^S). Mtz^R is relatively rare in anaerobes (Rasmussen *et al.*, 1997), and therefore, one might imagine that the high incidence of Mtz^R in microaerophiles is due to a mechanism of action that differs from that found in anaerobes. The available evidence from studies of protozoan and anaerobic bacterial species suggests that Mtz toxicity to *H. pylori* might depend on its reduction to the nitro anion radical and other compounds including hydroxylamine (Moreno *et al.*, 1982; Lindmark and Muller, 1975; Kedderis *et al.*, 1988). Hydroxylamine is particularly damaging to macromolecules such as DNA and proteins (Lindmark and Muller, 1976; Kedderis *et al.*, 1988). Under aerobic or

microaerobic conditions, molecular oxygen could convert reduced Mtz (i.e., the nitro anion radical) back to the parent compound by a process termed 'futile cycling', which essentially generates superoxide anions instead of hydroxylamine (Smith and Edwards, 1995). Because futile cycling has not been demonstrated experimentally (Smith and Edwards, 1995), reductions involving two and four electron transfers that favor hydroxylamine formation, such as would occur with ferredoxins and flavodoxins as electron donors, seemed very plausible, despite a lack of experimental evidence for direct enzymatic reduction of Mtz by *H. pylori.* Given this background, several possible mechanisms for Mtz^R in *H. pylori* merit consideration: decreased Mtz uptake or active efflux; deficiency in Mtz activation or modification; target modification or loss; and increased DNA repair or oxygen scavenging capabilities (Hoffman *et al.,* 1996). Indeed, inactivation of *recA,* a gene needed for generalized DNA repair and recombination, greatly enhances Mtz susceptibility of wild-type *H. pylori* (Thompson and Blaser, 1995); and cloned *recA* gene from a Mtz^R strain seems to increase the already very high level of resistance that *E. coli* exhibits (Chang *et al.,* 1997).

Thus a need exists for the identification of the gene(s) responsible for the Mtz^R and Mtz^S phenotypes in *H. pylori,* and characterization of proteins encoded by such gene(s).

BRIEF DESCRIPTION OF THE INVENTION

In accordance with the present invention, the gene responsible for metronidazole sensitivity in *H. pylori* has been identified. Mutational inactivation of the gene, which encodes an oxygen-insensitive NADPH nitroreductase, referred to herein as *rdxA* (designated HP0954 in the entire genome sequence) (Tomb *et al.,* 1997), is the cause of naturally acquired Mtz^R in *H. pylori.* In accordance with another embodiment of the present invention, there is provided a method of employing *RdxA* and related compounds, optionally in conjunction with targeting compounds, to convert nitroaromatic compounds to cytotoxins for use in selectively

killing or inhibiting the growth of target cell populations. In accordance with another aspect of the present invention, there is provided a method of employing RdxA and related compounds in order to convert nitroaromatic compounds to cytotoxins for use in selecting against cells expressing *rdxA*.

BRIEF DESCRIPTION OF THE FIGURES

Fig. 1. shows the nucleotide sequence and deduced amino acid sequence of *rdxA* of WT strain 500. The Shine-Dalgarno (SD) ribosome-binding site is underlined on the nucleotide sequence. The underlined amino acid sequence defines a highly conserved region among classic nitroreductase (CNR) proteins. Cysteine residues are highlighted in bold face and the *Sph*1 sites used for insertion of the *camR* cassette are underlined and noted. ***H. pylori* strains 439 and 1107 contain transition substitutions (TT for CC).

Fig. 2. indicates the location of amino acid substitutions in RdxA from matched $Mtz^{R/S}$ strains and from clinical isolates. *H. pylori* strain 1107 was created by transforming DNA from Mtz^{R} strain 439 into Mtz^{S} strain 500. Note that the RdxA amino acid sequence is identical, indicating allelic exchange recombination occurred outside the *rdxA* locus. Other clinical isolates are included for comparison. The five matched pairs of isolates are grouped separately and the amino acid substitutions are listed in Table 3.

DETAILED DESCRIPTION OF THE INVENTION

In accordance with the present invention there are provided novel nitroreductases, having two or more cysteine residues, an alkaline pI greater than about 6.0, a preference for NADPH as an electron donor, and having the ability to convert a prodrug to one or more cytotoxic compounds. Preferably invention nitroreductases have a pI of about 7.99.

As used herein, prodrug refers to compounds of the general structure $X\text{-}NO_2$, wherein X is an organic radical of structure sufficient to impart to $X\text{-}NO_2$ a low redox

potential. Preferably X-NO_2 has a redox potential in the range of about -500mV to about -350mV. Those of skill in the art will clearly recognize that a number of organic species are suitable for the X moiety, including, without limitation, pyrroles, furans, thiophenes, imidazoles, oxazoles, thiazoles, pyrazoles, pyridines, pyrimidines, purines, quinolines, isoquinolines, carbazoles, as well as substituted variants thereof. In one embodiment of the present invention, "prodrug" includes imidazoles, nitrofurazones, furanyls, and derivatives thereof such as nitroimidazoles, and the like. Preferred prodrugs include compounds used to treat Helicobacter infections such as metronidazole, nitazoxanide, and the like. An especially preferred prodrug is metronidazole. In a still another embodiment of the present invention, a prodrug is characterized by the ability to be converted to one or more hydroxylamines by action of invention nitroreductases.

In accordance with another aspect of the present invention, there are provided nitroreductases further characterized as being encoded by DNA having greater than about 90% homology with the *H. pylori rdxA* gene (see SEQ ID NO:1 and Fig. 1). Preferably, invention nitroreductases contain a conserved amino acid motif common to the CNRs (QPWHF) as well as the positioning of a strategic cysteine residue (position 87, see SEQ ID NO:2). In a more preferred aspect of this embodiment, invention nitroreductases are isolated from microaerophilic bacterial species such as *Helicobacter, Campylobacter,* and the like. An especially preferred nitroreductase is the *H. pylori* nitroreductase (RdxA) and homologues thereof. Those of skill in the art will readily recognize that similar nitroreductases can be isolated from other *Helicobacter* species, including, *H. acinonyx, H. bilis, H. bizzozeronii, H. canis, H. cholecystus, H. cinaedi, H. felis, H. fennelli, H. heilmanni, H. hepaticus, H. muridarum, H. mustelae, H. nemestrenae, H. pullorum, H. rodentium, H. salamonis, H. suncus, H. trogontum*, and the like. The presently preferred nitroreductase is the RdxA of *H. pylori* strain HP950.

In accordance with another aspect of the present invention, there are provided conjugates comprising a targeting compound and a nitroreductase, as defined herein.

In yet another aspect of the invention, there are provided conjugates wherein said targeting compound is covalently linked to a nitroreductase. As used herein, "covalently linked" refers to a bond between the targeting compound and nitroreductase wherein electrons are donated by one or more atoms of each to form the bond shared between the targeting compound and the nitroreductase. In a preferred aspect of the present invention, said targeting molecule is an antibody, to include monoclonal antibodies, and the like. Antibodies used in the present invention may be isolated and/or made with specificity cell surface antigens, precancerous cell surface antigens, cell surface antigens characteristic of autoimmune diseases (including for example, arthritis, Lupus, and other autoimmune diseases/conditions), tissue-specific antigens, organ-specific antigens, and the like. Those of skill in the art will readily recognize that antibodies, for use as targeting molecules may be generated with specificity to any cell population with characteristic antigenicity. Such antibodies, when conjugated with invention nitroreductases are contemplated embodiments of the present invention.

In accordance with another aspect of the present invention, there are provided nucleic acid molecules encoding the invention nitroreductases as defined herein. In a preferred embodiment of the present invention, said nucleic acid is greater than about 90% homologous to the *H. pylori rdxA* gene (see SEQ ID NO:1 and Fig. 1). In a presently preferred embodiment, the nucleic acid is homologous to the ORF shown in Fig. 1. In accordance with still another aspect of the present invention, said nitroreductase-encoding nucleic acid is expressed in a heterotypic cell. As used herein, "heterotypic cell" refers to a cell or virus other than that in which said nucleic acid is found in nature. Those of skill in the art will readily recognize that, with appropriate manipulation, the range of heterotypic cells in which invention nucleic acids can be expressed includes, bacteria, viruses, retroviruses, yeast, eukaryotic cells, and the like. Expression of invention nucleic acids in each of these cell types is contemplated by the present invention, as are the proteins so expressed.

In accordance with another aspect of the present invention, there are provided methods for selectively killing or inhibiting the growth of target cells, said method comprising administering invention conjugates in conjunction with administration of a prodrug, as defined herein, wherein said nitroreductase converts said prodrug into one or more cytotoxic compounds, resulting in the killing or growth-inhibition of the target cells. Preferably, target cells are selected from bacterial, (retro)viruses, fungi, yeast, immune system cells such as T-cells, and B-cells, tissue cell, organ cells, diseased cells, tumor cells or neoplastic cells.

In still another embodiment of the present invention, there are provided pharmaceutical formulations comprising the nitroreductase, or conjugated nitroreductase as defined herein. In another aspect of this embodiment, pharmaceutical formulations will include a suitable carrier. Those of skill in the art will recognize that, depending upon indications, mode of administration and the intended recipient/patient, formulations can include a variety of carriers. Suitable carriers contemplated for use in the practice of the present invention include carriers suitable for oral, intravenous, subcutaneous, transcutaneous, intramuscular, intracutaneous, inhalation, and the like administration. Administration in the form of creams, lotions, tablets, dispersible powders, granules, syrups, elixirs, sterile aqueous or non-aqueous solutions, suspensions or emulsions, patches, and the like, is contemplated.

For the preparation of oral liquids, suitable carriers include emulsions, solutions, suspensions, syrups, and the like, optionally containing additives such as wetting agents, emulsifying and suspending agents, sweetening, flavoring and perfuming agents, and the like.

For the preparation of fluids for parenteral administration, suitable carriers include sterile aqueous or non-aqueous solutions, suspensions, or emulsions. Examples of non-aqueous solvents or vehicles are propylene glycol, polyethylene glycol, vegetable oils, such as olive oil and corn oil, gelatin, and injectable organic

esters such as ethyl oleate. Such dosage forms may also contain adjuvants such as preserving, wetting, emulsifying, and dispersing agents. They may be sterilized, for example, by filtration through a bacteria-retaining filter, by incorporating sterilizing agents into the compositions, by irradiating the compositions, or by heating the compositions. They can also be manufactured in the form of sterile water, or some other sterile injectable medium immediately before use. Suitable carriers may also include liposomes, microspheres, or latex beads, and the like.

Invention compounds can optionally be converted into non-toxic acid addition salts. Such salts are generally prepared by reacting the compounds of this invention with a suitable organic or inorganic acid. Representative salts include the hydrochloride, hydrobromide, sulfate, bisulfate, methanesulfonate, acetate, oxalate, valerate, oleate, laurate, borate, benzoate, lactate, phosphate, tosylate, citrate, maleate, fumarate, succinate, tartrate, napsylate, and the like. Such salts can readily be prepared employing methods well known in the art.

In another embodiment of the present invention, there are provided methods for detecting plasmid loss by a bacterium, said method comprising transforming a bacterium with a plasmid containing DNA encoding invention nitroreductases as described herein, and assaying for growth of said bacteria on nitroaromatic-containing media, wherein said nitroreductase, as inserted into said plasmid, is expressed in said bacteria, and identifying as having lost the plasmid, any of said transformed bacteria which grow on said nitroaromatic-containing media.

In yet another embodiment, there are provided methods for identifying substrates for nitroreductases as defined hereinabove. Methods according to this embodiment comprise transforming a host cell with a plasmid encoding said nitroreductase, and assaying for growth of said host cell on a medium containing the putative substrate, wherein said nitroreductase is expressed and converts any substrate present in said medium to one or more cytotoxic compounds such that said transformed cells will be killed or growth-inhibited, and identifying as a substrate any

of said putative substrates causing killing or growth-inhibition of said transformed cells.

Also contemplated within the present invention is a kit for identifying whether a bacterial isolate expresses a nitroreductase as defined herein. Said kit comprising a substrate for said nitroreductase, wherein said nitroreductase converts said substrate into one or more detectable products, and a means for detecting said product(s).

Typically, bacteria contain several different nitroreductases including flavin and ferredoxin reductases that may exhibit nitroreductase activity (Zenno *et al.*, 1996a,b). One relatively close homologue of *rdxA*, with 25% protein-level identity over 181 amino acids, is *frxA* (HP0642), which encodes a NAD(P)H flavin reductase (FrxA) similar to the flavin reductase of *Haemophilus influenzei (Tomb et al.*, 1997). The results presented herein suggest that FrxA does not contribute significantly to Mtz susceptibility or resistance. In support of the latter hypothesis, it has also been discovered that the *frxA* gene cloned in the pBluescript plasmid vector does not affect the intrinsic high resistance of *E. coli* to Mtz. As even Mtz^R strains of *H. pylori* become susceptible to Mtz under anaerobic conditions (Smith and Edwards, 1995), perhaps FrxA and/or other ferredoxin and flavin reductases, such as those found in the annotation of the *H. pylori* genome sequence (Tomb *et al.*, 1997), may contribute to the activation of Mtz under anaerobic conditions.

Some investigations of metronidazole resistance focused on the metabolic enzymes of *H. pylori;* in particular, on pyruvate:ferredoxin/flavodoxin oxidoreductase (POR) and α-ketoglutarate oxidoreductase (KOR) (Hoffman *et al.*, 1996), in part because studies in anaérobes had shown POR to be responsible for Mtz activation (Moreno *et al.*, 1983; Narikawa, 1986 Lockerby *et al.*, (1991). Our studies showed that POR and KOR activities of Mtz^R strains of *H. pylori* were repressed in bacteria that had been cultured in the presence, but not in the absence, of Mtz (Hoffman *et al.*, 1996). This indicated that these reductases were regulated by Mtz, which is consistent with a model in which *H. pylori* POR and KOR mediate Mtz toxicity. However,

those experiments did not test whether this, or any of several other changes that have been identified to date (see Hoffman *et al.*, 1996; Smith and Edwards, 1997), is a primary effect, and the cause of resistance, or a secondary consequence of other metabolic perturbations that Mtz elicits. Similarly, although Mtz^R mutants are easily derived from many Mtz^S strains in the laboratory, the genetic basis for naturally occurring resistance, whether mutation in a normal chromosomal gene or by acquisition of a new 'resistance' gene, was unknown.

The basis of susceptibility and resistance to the antimicrobial agent metronidazole (Mtz) in *H. pylori* has been examined. Experiments indicate (i) that the toxicity of Mtz for *H. pylori* likely depends on its reduction to hydroxylamine by an oxygen-insensitive, chromosomally encoded NADPH nitroreductase (*rdxA*; HP0954 in the genome database) (Tomb *et al.*, 1997); (ii) that resistance results from mutational inactivation of *rdxA* and not from the acquisition of foreign resistance genes (in contrast to common mechanisms of resistance against other antibiotics and bacterial species) (Levy, 1992). Mtz^R strains display no significant changes in metabolic or growth capacity compared with isogenic Mtz^S strains in culture (Hoffman *et al.*, 1996).

Four results established the importance of a functional *rdxA* gene in Mtz^S, and *rdxA* inactivation as the general mechanism of Mtz^R in *H. pylori*. First, a mutant allele of *rdxA was* found using a DNA transformation strategy: one cosmid in a library made from a Mtz^R clinical isolate was found to transform a Mtz^S recipient to Mtz^R; subcloning from this cosmid, and further transformation identified the segment responsible for Mtz^R, and DNA sequencing revealed *rdxA*, a nitroreductase gene with significant protein level homology to the CNRs of enteric bacteria. The allele of *rdxA* that was responsible for transformation of the Mtz^S strain to Mtz^R in these first experiments contained a nonsense (translational stop) codon 14 codons before the 3' end of the ORF (as defined by sequences of *rdxA* genes from Mtz^S strains). Second, *E. coli*, which is normally Mtz^R, was rendered Mtz^S by cloned *rdxA* genes from each to of 8 Mtz^S *H. pylori* strains, but not by cloned *rdxA* genes from any of 8 Mtz^R strains

contain mutant (inactive) *rdxA* genes. DNA sequencing showed that point mutations (missense and nonsense) at other sites in *rdxA* were responsible for *rdxA* inactivation in these strains. Third, introduction of *rdxA* from a Mtz^S *H. pylori* strain on a shuttle vector plasmid rendered a formerly Mtz^R recipient strain Mtz^S, this further illustrates that a functional RdxA nitroreductase contributes to the Mtz^S phenotype of normal *H. pylori*. Fourth, *H. pylori* derivatives with *camR* inserts in their *rdxA* genes, and that had been selected solely by their Cm^R phenotype, exhibited a typical Mtz^R phenotype. Collectively, these results showed that a functional RdxA nitroreductase is key to the normal Mtz^S phenotype of wild-type *H. pylori*, and, conversely, that *rdxA* inactivation is necessary and sufficient for Mtz^R in this species.

It is believed that the multiple cysteine residues of RdxA together with the more alkaline nature of the protein may contribute to both a lower redox potential and a greater substrate specificity of this enzyme for Mtz. These properties might be achieved through the formation of disulphide bonds or the chelation of metal cofactors, which might form a flavin-independent catalytic center. It has been suggested that a disulphide bond of the CNR homodimer may participate as an electron acceptor in the oxidation of NAD(P)H (Inouye, 1994; but see Zeno et al., 1996a) and in an alkyl hydroperoxide reductase from S. typhimurium, two cysteine residues participate in catalysis (Ellis and Poole, 1997).

In studies of H. pylori from human populations at high risk of infection (Peru, Lithuania), pairs of strains have been identified, one Mtz^R and one Mtz^S, that were closely matched in RAPD fingerprint. Although rdxA genes from unrelated strains differed in DNA sequence by 5% on average, the rdxA genes from Mtz^S and Mtz^R isolates from the same person differed by only one or a few base substitutions. This result indicated that Mtz^R resulted from de novo mutation, and not by gene transfer from an unrelated Mtz^R strain, even although at least transiently mixed infection seems to be quite common in these high risk (Peruvian and Lithuanian) societies.

Nitroreductases from other organisms are classified as oxygen sensitive or insensitive based on whether the substrates are reduced in one- or two-electron transfer reactions respectively. One-electron transfer reductions of the nitro group of a particular compound produces the nitro-anion radical, which in the presence of oxygen generates superoxide anions and regeneration of the 5-nitro group (Moreno *et al.*, 1983; Edwards, 1993). It has been suggested that aerobes and facultative anaerobes are resistant to Mtz because under aerobic conditions redox cycling leads to regeneration of Mtz (Smith and Edwards, 1995). Indeed, the Mtz^S of *Actinobacillus actinomycetemcomitans* under anaerobic, but not aerobic conditions, is consistent with the concept of redox cycling and the nitroreductase activity implicated in Mtz^S of this species may be of the oxygen-sensitive type (Pavicic *et al.*, 1995). In contrast, the Mtz^S of *H. pylori* was not affected by growth under different oxygen tensions; suggesting that one electron transfer is probably not involved in Mtz reduction in this microaerophilic bacterium (Smith and Edwards, 1995). The latter interpretation is supported by the present finding that an oxygen-insensitive nitroreductase is responsible for the Mtz^S of *H. pylori*. Microaerophiles in general are susceptible to Mtz, and display patterns of resistance similar to those noted for H. pylori (Hoff and Stricht-Groh, 1984; Lariviere et al., 1986), suggesting that homologues of rdxA may be found in these other species.

Naturally occurring Mtz^R is associated with a Mtz-inducible depression of activity of pyruvate oxidoreductase (POR) and as little as 3-5μgml^{-1} of Mtz in the culture medium is sufficient to abolish POR activity of Mtz^R strains (Hoffman, et al., 1996). This depression of POR was also seen in a Mtz^R strain containing a camR insertion in rdxA, a strain selected by its Cm^R, not by its Mtz^R. This result indicates that repression of POR activity is not due to a secondary mutation selected by enhancement of Mtz^R. Based on studies with anaerobes, POR should also be capable of reducing Mtz (Lockerby et al., 1985), and it is proposed that the POR of H. pylori acts similarly. This thinking suggests that POR activity could be responsible for the transient growth inhibition and limited killing seen when Mtz^R H. pylori are first exposed to Mtz (Lacey et al., 1993). The ability to turn off synthesis or accumulation

of POR in response to Mtz might then be an important component of resistance. Just how this putative regulatory mechanism operates is not yet known, but it is attractive to imagine that it involves a response to the chemical (hydroxylamine induced) damage to DNA, protein or other macromolecules, analogous to the bacterial response to alkylation damage (see Volkert, 1988, 1989). Such a mechanism might also be advantageous during normal growth (without Mtz treatment), helping safeguard H. pylori against deleterious effects of reduction of other nitroaromatic compounds that might be encountered in situ such as hydroxylamine adducts that might result from the action of nitric oxide with amines.

The majority of nitroreductases thus far studied are of the oxygen-insensitive type and are capable of reducing nitroaromatic compounds through sequential two-electron reductions, resulting in nitroso intermediates and hydroxylamine end products (Lindmark and Muller, 1976; Bryant and Deluca, 1991). This interpretation is supported by the direct demonstration that the enteric homologues of RdxA (CNRs NfsB of *E. coli,* Cnr of *Salmonella typhimurium,* and NfsB of *E. Cloacae)* reduce 4- and 5-nitro compounds by two-electron transfer reactions (Bryant and Deluca, 1991; Zenno *et al.,* 1996a;, Yamada *et al.,* 1997). The substrate specificity of the CNRs is often a function of the redox potential of the 5-nitro group (Bryant and Deluca, 1991), and in this regard the intrinsic resistance of enteric bacteria to Mtz is due to the very low redox potential of Mtz (Narikawa, 1986). However, reduction of Mtz and other nitroaromatic compounds to mutagenic end products by *S. typhimurium* has been demonstrated in the Ames test (Lindmark and Muller, 1976). Null mutations in the *S. typhimurium* gene for Cnr, an *rdxA* homologue, renders *S. typhimurium* resistant to the mutagenic effects of nitro-containing compounds (Yamada *et al.,* 1997). It appears that CNR activates Mtz in these microbes, generating hydroxylamine at levels that are too low to cause much lethality yet are still sufficient for mutagenesis. The Mtz^S of *E. coli* strains containing cloned *H. pylori rdxA* genes, for which Mtz reductase activity was measured in two strains, suggests that lethality must be due to the greater production of hydroxylamine from Mtz by the *H. pylori* RdxA nitroreductase.

The invention will now be described in greater detail by reference to the following non-limiting examples.

EXAMPLES

Example 1: *Identification of a nitroreductase that confers Mtz sensitivity in* H. pylori

The gene responsible for naturally occurring Mtz^R in *H. pylori* was sought using a strategy based on an earlier finding (Wang *et al.*, 1933) that DNA from Mtz^R clinical isolates could transform Mtz^S strains to Mtz^R. To maximize the chance of finding the Mtz^R determinant, independent of whether naturally occurring Mtz^R is caused by a particular type of allele of a normal chromosomal gene, or by an added gene that is absent from the genomes of Mtz^S strains, a cosmid cloning approach was employed.

Bacterial strains and growth conditions

The *H. Pylori* isolates used in this study were isolated from human gastric biopsy samples and were obtained from the Victoria General Hospital, Halifax, Nova Scotia, Canada, and have been previously described (Hoffman *et al.*, 1996). Paired Mtz^R and Mtz^S from the same patient that were found to be closely matched in overall genotype had been isolated from biopsies from Peruvian and Lithuanian patients, which were kindly provided by Drs. R. H. Gilman and H. Chalkauskas respectively. Bacterial strains were grown at 37°C on Brucella agar plates supplemented with 10% fetal calf serum (FCS) in a microaerobic incubator maintained at 7%O_2, 5% CO_2. Liquid cultures were grown in Brucella broth with 10% FCS in 125ml screw-capped flasks; the medium was equilibrated with 7%O_2, 5% CO_2 in the microaerobic incubator for 1 h before inoculation, and then the flasks were sealed and placed on a rotary shaker at 150r.p.m. Unless otherwise indicated, metronidazole-resistant strains were grown with 18μgml^{-1} of Mtz, which is one half the minimal inhibitory concentration. Bacteria were harvested by centrifugation after 3-4 days of growth,

and either used immediately or stored as a pellet at -70°C. *E. coli* strains DH5α (BRL) and ER1793 (New England Biolabs) were grown on Luria-Bertani (LB) agar plates supplemented with the appropriate antibiotics.

Cosmid library construction and screening

Genomic DNA was prepared from Mtz^R strain 439 and partially digested with *Sau3A* to generate a population of DNA fragments in the 20-45kb range, as described previously (Hoffman *et al.*, 1989). These DNA fragments were cloned into *Bam*HI-cleaved Lorist6, a cloning vector that has been useful for making cosmid libraries from other *H. pylori* strains, and the ligated DNA was packaged into λ phage particles (Bukanov and Berg, 1994). Cosmids were recovered after infection of *E. coli* ER1793, which is deficient in restriction/modification systems, and transductants carrying cosmid clones were selected on LB agar containing 3Oμgml^{-1} of kanamycin. Kan^R colonies were picked into wells in microtitre dishes. Cosmid DNAs were prepared in batches from the growth of 96 clones per microtitre plate in 50ml of LB broth and the cosmid

Natural transformation and isolation of spontaneous metronidazole-resistant mutants

Transformation of Mtz^S and Mtz^R was carried out using a modification of the method of Wang *et al.* (1993), as follows. Log phase recipient cells (strains 500 or 1134) were prepared in 10ml of broth from overnight culture in Brucella broth. The bacterial pellet was resuspended in 0.5ml of TE (Tris EDTA) buffer, and the suspension was spotted onto Brucella agar plates supplemented with 10% FCS. After 3-4h incubation, 3-8μg of chromosomal, cosmid or plasmid DNA was spotted onto the bacterial growth followed by incubation for 12-16h. The bacteria were scraped from the agar surface and suspended in a minimal volume of TE and aliquots were then spread on Brucella agar containing 18μgml^{-1} metronidazole. Transformed colonies were isolated from these plates after 3-4 days' incubation. Spontaneous

Mtz^R mutants were isolated by spreading 0.10ml of turbid cultures ($5x.10^9$ cells) on Brucella agar containing between 8 and 18μgml-^{1} Mtz.

Low-level transforming activity was found reproducibly in one of the nine pools tested (11 transformant colonies, vs. 15 in a control using 15 μg of strain 439 genomic DNA). No transformants were obtained with cosmid DNAs from any of eight other microtitre plates. The cosmid responsible for Mtz^R-transforming activity was identified in two more transformation steps: first using 12-member pools prepared from each of the eight rows in this microtitre plate; and then using individual cosmids from the one active row.

Details of DNA subcloning and sequencing

*Eco*Rl digestion generated four DNA fragments from the cosmid containing the Mtz^R determinant, and the Mtz^R-transforming activity was localized to one of them, a 2.3kbp fragment. DNA sequencing was carried out on both strands, manually using the Sequenase kit (Amersham) or by automated methods on a Licor DNA sequencer at the Institute for Marine Biosciences facility of the National Research Council of Canada (Halifax, NS). The sequence was assembled and analyzed using the Wisconsin Group GCG software (Devereau *et al.,* 1984) and BLAST search routines to assist identification of ORFs and other sequence features.

Two open reading frames (ORFs) were found in the 2.3kbp fragment. One ORF (corresponding to HPO955 in the entire *H. pylori* genome sequence, (Tomb *et al.,* 1997)) had strong protein-level homology to the gene for prolipoprotein diacyglycerol transferase *lgt* and seemed unlikely to be involved in Mtz^R. The second ORF had protein-level homology to classical oxygen-insensitive NAD(P)H nitroreductases (CNRs) of several other Gram-negative bacteria (see Table 1) and was a good candidate because some of its homologues are known to reduce metronidazole or related compounds (Lindmark and Muller, 1976; Yamada *et al.,* 1997). This *H. pylori* gene corresponds to the ORF designated HP0954 in the full genome sequence (Tomb *et al.,* 1997) and, interestingly, exhibits 54% similarity with another

ORF (HP0642) that encodes a NAD(P)H flavin nitroreductase (*'frxa'* herein), also a CNR homologue. The sequences have been deposited in GenBank (AFO12552, AFO12553).

Table 1. Similarity of RdxA to other classical nitroreductases.

Bacterial Strains	Protein	Per cent Identity	Per cent Similarity
Haemophilus influenza	NtsB	25	48
Enterobacter cloacae	NfnB	30	50
Salmonella typhimurium	Cnr	30	50
Helicobacter pylori	FrxA	27	54
Escherichia coli	NfsB	28	49

The inferred RdxA product from Mtz^R *H. pylori* strain 439 is 196 amino acids long. PCR amplification and sequencing of the corresponding segment from the Mtz^S strain 500 revealed an ORF that is 14 codons longer at the 3' end (210 codons, see Fig. 1). The *rdxA* gene from a Mtz^R transformant of strain 500 (strain HP 1107) that was made with genomic DNA from strain 439 was identical in DNA sequence to that of the 439 parent strain (Fig. 2). These results indicate that Mtz^R *H. pylori* can result from inactivation of *rdxA*, which in strain 439 occurred by a nonsense mutation that resulted in a truncated RdxA protein.

The WT *rdxA* gene was 630bp in length and had a Shine-Dalgarno sequence 5bp upstream of the start codon. The CNR proteins of the enteric bacteria are acidic proteins, including HP0642 *('frxA')* (pI=5.4-5.6), and generally contain one to two cysteine residues. However, RdxA is a basic protein (pI=7.99) and contains six cysteine residues. One of the cysteine residues (position 87) is conserved in the CNR

proteins of the enterics. The cysteine located at position 159 is in a motif (L/IDSCI/PI) shared with the inferred product of *frx*A. Another motif common to all of the CNRs is QPWHF (PW is absolutely conserved) located within a highly conserved region between positions 43-59 in RdxA.

Example 2: *Nitroreductase activity and rdxA expression in* E. coli

Cell-free extracts from Mtz^S and Mtz^R strains of *H. pylori* were screened for nitroreductase activity using standard assays that use either menadione or nitrofurazone as electron acceptors (Bryant and Deluca, 1991; Zenno *et al.*, 1994) (data not presented). No significant differences in the nitroreductase activities of either isogenic pairs of Mtz^S and Mtz^R strains or of various clinical isolates were detected, suggesting that *H. pylori* most probably possesses multiple nitroreductases. The latter hypothesis is supported by known genes present in the full genome sequence (e.g., *frxA* (H0642), (Tomb *et al.*, 1997)), and by the fact that multiple nitroreductases have been found by others in enteric bacteria (Zenno *et al.*, 1996a,b). No Mtz reductase activity was detected in crude extracts from Mtz^S strains of *H. pylori,* independent of whether NADPH or NADH were used as electron donors; this is consistent with earlier observations (Hoffman *et al.*, 1996). The inability to detect Mtz reductase activity in cell-free extracts of *H. pylori* might be attributable to oxidation of key components during the preparation, or to an inability of the assays used to detect very low levels of Mtz reductase activity.

Because *E. coli* strains are intrinsically resistant to Mtz (>300 μgml^{-1}), the possibility that expression of *rdxA* in *E. coli* might render the organism susceptible to Mtz was explored. It was found that the cloned r*dxA genes (rdxA* cloned in a pBluescript vector, downstream of the *lac* promoter) from each of 8 Mtz^S *H. pylori* strains, indeed rendered *E. coli* Mtz^S (killing by 10-60 μgml^{-1}) during aerobic growth on LB agar. In contrast, equivalent plasmid clones made with *rdxA* genes from each of eight Mtz^R *H. pylori* had no effect on the intrinsic high level of Mtz resistance of the *E. coli* host.

Recombinant rdxA screen for Mtz$^{R/S}$

E. coli DH5α containing pBluescriptSK*rdxA* clones from all *H. pylori* strains used in this study were screened for Mtz^S on Luria Bertani medium containing a range of Mtz concentrations from 0 to 60μgml-^{1}. The plates were streaked for isolation of colonies or a 1:1:00 dilution of a 0.40D_{660} broth culture was spread onto the medium. The plates were incubated under aerobic conditions at 37°C and then scored for growth at 16-24h.

Each of the strains used in the *rdxA* sequence analyses (Fig. 2) was tested in this way, yielding results that completely supported the use of *in vivo* assays in *E. coli* as a surrogate for monitoring the *rdxA* activity of *H. pylori. An in vivo* assay of *frxA* (cloned from the 26695 strain of *H. pylori* into pBluescript) in *E. coli* indicated that the FrxA (flavin reductase) activity did not alter the intrinsic resistance of *E. coli* to Mtz.

The cloned *rdxA* gene from the *H. pylori* strain that rendered *E. coli* most susceptible to Mtz (strain 950) was tested for nitroreductase activity by spectrophotometric assay. Cell-free extracts from *E. coli* harboring *rdxA* from this strain exhibited 40-fold higher than background NADPH-dependent nitroreductase activity using metronidazole as the electron acceptor, and assayed by following either Mtz reduction or oxidation of NADPH (Table 2). No detectable reductase activity was found using NADH instead of NADPH as the electron donor, nor was any detected using extracts of *E. coli* carrying pBluescript by itself or with an *rdxA* mutant (Mtz^R allele from strain 1043). These results indicate that RdxA protein can reduce Mtz and differs from other CNRs in showing specificity for NADPH. Among the known nitroreductases, only NfsA of *E. coli* shows specificity for NADPH (Zenno *et al.,* 1996b), but this gene exhibits no DNA- or protein-level homology with RdxA (or with FrxA, HPO642) of *H. pylori.* These results indicate that expression of WT *rdxA,* but not *frxA* in *E. coli;* causes a marked increase in susceptibility to Mtz and

support the conclusion that *rdxA* function is responsible for the Mtz^S of wild-type *H. pylori*, and that Mtz^R in this pathogen results from *rdxA* inactivation.

Table 2. Metronidazole reductase activity of RdxA nitroreductase.

	nmol min^{-1} mg^{-1} protein	nmol min^{-1} mg^{-1} protein
Isolate	Mtz (A_{320})	NADPH (A_{340})
pBSK	0.09	0.62
pBS950	9.23 +/-0.87	14.13 +/- 0.70
pBS1043	0.31	0.40

Metronidazole reduction was measured in crude extracts of *E. coli* strain JF626 grown aerobically in LB broth. pBSK is pBluescript vector control; pBS950 is WT *rdxA* cloned into pBSK and pBS1043 is MtzR *rdxA* cloned in pBSK. The assay contained NADPH and Mtz. The enzymatic reaction was followed at 320 nm to measure Mtz reduction and at 340 nm to measure NADPH oxidation. The values are corrected for NADPH oxidase activity. No activity was found when NADH was used as substrate.

Enzyme assays

Cell-free extracts were prepared from bacteria that had been grown to mid to late log phase in the appropriate medium and where indicated, either in the presence or absence of 18μgml^{-1} Mtz. The general protocol for preparation of cell-free extracts has been previously described (Hoffman *et al.*, 1996). All enzyme assays were

carried out at 25°C in 1 ml volumes in a modified Cary-14 Spectrophotometer equipped with an OLIS data acquisition system (On Line). Nitroreductase activity was assayed with NADH or NADPH at 340 nm (extinction coefficient, 6.22 mM^{-1} cm^{1}) or by following the reduction of metronidazole at 320 nm (E=9.2mM^{-1} cm^{-1}). The reaction mixture contained Tris/acetate (100mM Tris-HCl, 50mM acetate) pH 7.0, 0.05mM Mtz and 0.3mM NADPH or HADH, POR (EC 1.2.7.1) was assayed under anaerobic conditions with 74mM potassium phosphate (pH 7.3), 10mM sodium pyruvate, 5mM benzyl viologen, 0.18mM coenzyme A (CoA), and 5µM thiamine PP as described previously (Hoffman *et al.*, 1996). Reduction of benzyl viologen was followed at 546nm and specific activity was determined for the reaction using an extinction coefficient of 9.2mM^{-1} cm^{01}. Specific activities were reported as nmoles per min per mg of protein. Protein determinations were performed using the Bradford procedure (Bio-Rad) with bovine serum albumin as the standard.

Example 3: *Sequence analysis of* rdxA *in closely related pairs of* Mtx^R *and* Mtz^S *strains*

To assess how often Mtz^R is acquired by *de novo* mutation vs. *rdxA* gene transfer from an unrelated strain that is already Mtz^R, *rdxA* genes from infections that were mixed with respect to Mtz^R/Mtz^S, and in which the Mtz^R and Mtz^S isolates seemed to be very closely related based on arbitrarily primed PCR cloning/sequencing have been studied. *rdxA* sequences from various strains of *H. pylori* were amplified and cloned into pBluescript using primer pairs Mtz6EF (forward) 5'-TGAATTCGAGCATGGGGCAG and reverse primer Mtz^RBgl 5'-AGCAGGAGCATCAGATAGATCTGADNA.

With each of five such pairs of isolates studied, the PCR amplified *rdxA*-containing segment obtained was about the same size (≈937 bp). This implied that resistance was due to point mutations and not to insertion, deletion or other rearrangement. DNA sequence analysis showed that the *rdxA* genes from Mtz^R and Mtz^S members of each pair were closely related but differed by 1-3 bp in the 630-bp-

long gene (resulting in one or two amino acid replacements) in each case (see Fig. 2 and Table 3). Because unrelated *rdxA* genes differed on average by about 5% (28-34 bp of 630 bp), this indicates that Mtz^R was due to *de novo* mutation, not horizontal gene transfer from another strain.

Table 3. Types of point mutations in matched pairs of Mtz^R and Mtz^S strains and

Strain	A-G	C-T	Other	Amino Acid Substitution
H2amt	1			Arg-Gly
B1amt	3			Tyr-Cys, Ala-Thr
21cmt	2			Gln-Arg, Lys-Glu
12mtz	1			Ala-Thr
10amt3	1	1	1	Gly-Val
439/500[a]	8	15	4	(8aa)

[a]Comparison of divergence in rdxA of unrelated *H. pylori* strains 439 and 500. Listed are the number of amino acid changes between these strains.

Four of the five alleles resulted in single amino acid changes in the inferred 210-amino-acid -long RdxA protein:G—V at position 145 in mutant 10amt3; A—T at position 180 in 12mtz; R—G at position 200 in H2mt; and K—E at position 63 in strain 21cmt. The fifth *rdxA* mutant allele (Blamt) would encode a protein with two amino acid sequence changes, Y—C at position 47, which is in a region that is highly conserved at CNRs (position 43-57), and also A—T at position 143.

Example 4: rdxA-*inactivation is sufficient for Mtz^R: allelic exchange mutagenesis and complementation*

Based on finding non-functional *rdx*A alleles in each Mtz^R clinical isolate studied, it was tested whether *rdxA* inactivation is also sufficient for resistance, or whether additional mutations are also needed.

pDH26, a chimeric shuttle vector, was kindly provided by Dr. Rainer Haas. *H pylori* strain 500 sequences spanning the *rdxA* ORF were excised from pBluescript by *Eco*RV and *Sal*I digestion and subcloned into similarly restricted pDH26. *H. pylori* strain 1061 was made MtzR by natural transformation of pBluescriptSK*rdxA* originating from Mtz^R strain 439. The pDH26*rdxA* plasmid was introduced into strain 1061Mtz^R by natural transformation and Cm^R colonies were scored on BA supplemented with 15μgml^{-1} of CM. CM^R colonies were subsequently screened for Mtz^S phenotype on Brucelia agar containing CM and 18μgml^{-1} Mtz to demonstrate dominance of wild-type *rdxA* through loss of the Mtz^R phenotype.

Allelic exchange mutagenesis and complementation

A 937bp PCR amplicon of *H. pylori Mtz^S* strain H2csr, generated with oligonucleotide primers Mtz6EF and Mtz^{R}Bgl and cloned into pBluescript-SK (a non-replicating vector), was digested with *SphI,* which deleted an approximately 160bp fragment from an internal region of *rdxA* (see Fig. 1 for *SphI* sites). After gel purification and generation of blunt ends with T4DNA polymerase, an *Eco*RV restricted *cam* cassette originating from *Campylobacter coli* (Wang and Taylor, 1990) was ligated into *rdxA* to create *pBluescriptrdxA::cam.* After transformation into DH5α and plasmid purification, *pBluescriptrdxA::cam* was introduced into Mtz^S *H. pylori* strain 26695 by natural transformation. Cm^R colonies were picked and then scored for Mtz^R. Each of the 30 Cm^R transformants tested was able to grow on Mtz-containing medium (18 μg ml^{-1} Mtz), and thus had acquired high-level Mtz^R. This showed that simple inactivation of *rdxA* is sufficient for Mtz^R in *H. pylori.*

Previous studies had shown that growth of Mtz^R strains in Mtz-containing medium resulted in disappearance of POR activity, another enzyme that putatively can reduce Mtz, and therefore that should render *H. pylori* Mtz^S whenever it is active (Hoffman *et al.*, 1996). In the present experiments, it was determined that growth of the *rdxA::camR* insertion mutant strain (which had been selected solely by its Cm^R phenotype) in Mtz-containing medium also resulted in the disappearance of POR activity. In addition, during growth in Mtz-free medium, this mutant strain exhibited only half as much POR activity as its isogenic *rdxA*$^+$ (Mtz^S) parental strain. Thus, mutations in *rdxA* may indirectly affect the level of POR activity through a potentially important mechanism.

In complementary experiments, the *rdxA* gene from the Mtz^S strain 500 was PCR amplified and cloned into pDH26, a Cm^R shuttle vector that is stably maintained in *H. pylori* (obtained from R. Haas), and the construct was transformed into the Mtz^R strain 1061R. Strain 1061 had been made Mtz^R by transformation of a mutant *rdxA* allele originating from Mtz^R strain 439. Each of the eight Cm^R colonies tested exhibited a Mtz^S phenotype, and *rdxA*-containing plasmid DNAs were easily reisolated from each of them; this indicates that the *rdxA* nonsense mutant allele is recessive, as expected. These results further establish that null mutations in just a single gene, *rdxA,* are responsible for Mtz^R in *H. pylori.*

While the invention has been described in detail with reference to certain preferred embodiments thereof, it will be understood that modifications and variations are within the spirit and scope of that which is described and claimed.